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<p>(54) Title: <b>CONSTITUTIVELY ACTIVE G PROTEIN COUPLED RECEPTOR OF HHV 8 AND METHOD</b></p> <p>(57) Abstract</p> <p>The present invention is directed to a constitutively active G protein coupled receptor of human herpesvirus 8, as well as a method of identifying negative antagonists of a constitutively active G protein coupled receptor. The method comprises co-expressing in a host cell a constitutively active G protein coupled receptor and a reporter protein, wherein expression of the reporter protein is controlled by a promoter responsive to a signalling pathway activated by the constitutively active G protein coupled receptor; exposing the host cell to a test substance; and determining a level of reporter protein activity, wherein the level of reporter protein activity indicates effectiveness of the test substance as a negative antagonist of the constitutively active G protein coupled receptor. The invention further provides a method of preventing tumor formation or cell proliferation caused by a constitutively active G protein coupled receptor. This method comprises administering an amount of the negative antagonist so identified to a subject in an amount effective to prevent tumor formation or cell proliferation.</p>			

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CONSTITUTIVELY ACTIVE G PROTEIN COUPLED RECEPTOR  
OF HHV 8 AND METHOD

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FIELD OF THE INVENTION

The present invention relates to a constitutively active G protein coupled receptor of human herpesvirus 8. The invention further relates to a method of identifying negative antagonists of constitutively active G protein coupled receptors, and to a method of preventing tumor formation or cell proliferation caused by the constitutively active G protein coupled receptor by administering an effective amount of the identified negative antagonist.

BACKGROUND OF THE INVENTION

Throughout this application various publications are referenced, many in parenthesis. Full citations for these publications are provided at the end of the Detailed Description of the Invention. The disclosures of these publications in their entireties are hereby incorporated by reference in this application.

The family of guanine nucleotide-binding (G) protein-coupled receptors (GPCRs) has been estimated to be comprised of as many as 2,000 members, fully more than 1.5% of all the proteins encoded in the human genome, that are thought to regulate function of virtually every cell in the body. Furthermore, it has been estimated that more than 50% of the drugs in use clinically in humans at the present time are directed at GPCRs.

In general, GPCRs require agonist binding for activation. Constitutive (or agonist-independent) signalling activity in mutant receptors has been well documented but only a few GPCRs have been shown to

5 exhibit agonist-independent activity in the wild type (or native) form. For example, native dopamine D1B and prostaglandin EP1b receptors have been found to possess constitutive activity (Tiberi and Caron 1994; Hasegawa et al. 1996). A number of GPCRs, for example, receptors for

10 thyroid-stimulating hormone (Vassart et al. 1995), have been found to be mutated to exhibit agonist-independent activity and cause disease in humans. Experimentally, several single amino acid mutations have produced agonist independent activity.  $\beta 2$  and  $\alpha 2$  adrenergic receptors, for example, mutated at single sites in the third cytoplasmic loop show constitutive activity (Ren et al. 1993; Samama et al. 1994). In some cases, a large deletion mutation in the carboxy tail or in the intracellular loops of GPCRs has led to constitutive

15 activity. For example, in the thyrotropin releasing hormone receptor a truncation deletion of the carboxyl terminus renders the receptor constitutively active (Nussenzveig et al. 1993; Matus-Leibovitch et al. 1995) and a smaller deletion in the second extracellular loop of the thrombin receptor causes constitutive activity (Nanevicz et al. 1995) also.

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The finding that certain receptors exhibit constitutive activity has led to a modification of traditional receptor theory (Samama et al. 1993). It is now thought that receptors can exist in at least two conformations, an inactive conformation (R) and an activated conformation (R\*), and that an equilibrium exists between these two states that markedly favors R over R\* in the majority of receptors. In some native receptors and in the mutants described above, it has been

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proposed that in the absence of agonist there is a shift in equilibrium that allows a sufficient number of receptors to be in the active R\* state to initiate signalling.

5 As many more GPCRs with constitutive activity are found, both native as well as mutated receptors, a newly recognized class of drugs termed negative antagonists (or inverse agonists) will become important therapeutic agents. Negative antagonism is demonstrated  
10 when a drug binds to a receptor that exhibits constitutive activity and reduces this activity. Negative antagonists appear to act by constraining receptors in an inactive state (Samama et al. 1994). Although first described in other receptor systems  
15 (Schutz and Freissmuth 1992), negative antagonism has been shown to occur with guanine nucleotide-binding (G) protein-coupled receptors (GPCRs), for example, opioid (Costa and Herz 1989; Costa et al. 1992),  $\beta_2$ -adrenergic (Samama et al. 1994; Pei et al. 1994; Chidiac et al.  
20 1994), serotonin type 2C (Barker et al. 1994), bradykinin (Leeb-Lundberg et al. 1994), and D1B dopamine receptors (Tiberi and Caron 1994). Thus, a technology for identifying negative antagonists of native and mutated GPCRs has important predictable as well as not yet  
25 realized pharmaceutical applications. Furthermore, because constitutively active GPCRs are tumorigenic, the identification of negative antagonists for these GPCRs can lead to the development of anti-tumor and/or anti-cell proliferation drugs.

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SUMMARY OF THE INVENTION

To this end, the subject invention provides a constitutively active G protein coupled receptor of human  
35 herpesvirus 8, and a method of identifying negative

antagonists of a constitutively active G protein coupled receptor. The method comprises co-expressing in a host cell a constitutively active G protein coupled receptor and a reporter protein, wherein expression of the 5 reporter protein is controlled by a promoter responsive to a signalling pathway activated by the constitutively active G protein coupled receptor; exposing the host cell to a test substance; and determining a level of reporter protein activity, wherein the level of reporter protein 10 activity indicates effectiveness of the test substance as a negative antagonist of the constitutively active G protein coupled receptor.

The invention further provides a method of preventing tumor formation or cell proliferation in a 15 subject caused by a constitutively active G protein coupled receptor. This method comprises identifying a negative antagonist of a constitutively active G protein coupled receptor; and administering to the subject an amount of the negative antagonist effective to prevent 20 tumor formation or cell proliferation caused by the constitutively active G protein coupled receptor.

#### BRIEF DESCRIPTION OF THE DRAWINGS

25 These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

30 Fig. 1 illustrates the putative two-dimensional topology of the HHV 8 GPCR;

Figs. 2 and 3 illustrate competition binding of human recombinant  $^{125}\text{I}$ -IL-8 by unlabelled chemokines to COS-1 cells transfected with pcKSHV GPCR (10 mg/ml). (2) C-X-C chemokines ( $K_d$  or  $K_i$ ): IL-8 (25 nM), NAP-2 (23 nM), 35 PF-4 (150 nM) and MGSA (270 nM). (3) C-C chemokines ( $K_d$  or

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$K_i$ ): I-309 (110 nM) and RANTES (520 nM). Data represent the mean of triplicate determinations in a representative of two experiments;

5 Figs. 4 and 5 illustrate the characterization of inositol phosphate (IP) accumulation in COS-1 cells transfected with pcKSHV GPCR. (4) Comparison with COS-1 cells transfected with plasmids for human IL-8 receptors (type A or B; 10 mg/ml) in the presence or absence of plasmid encoding  $G\alpha_{16}$  (2 mg/ml) after 90 minutes. (5) 10 Time course in COS cells transfected with 10 mg/ml pcKSHV GPCR. Data represent the mean +/- S.D. of triplicate determinations in a representative of three experiments; IP accumulation of mock-transfected, untreated cells has been subtracted from all points;

15 Fig. 6 illustrates the dose dependence of inositol phosphate (IP) accumulation in COS-1 cells after transfection with various amounts of pcKSHV GPCR (from 0.003 to 10 mg/ml), measured at 90 min. Data represent the mean +/- S.D. of triplicate determinations in a 20 representative of three experiments;

25 Fig. 7 illustrates the transcription of luciferase driven by a PKC-responsive promoter in COS-1 cells transfected with pcKSHV GPCR. Data represent the mean +/- S.D. of triplicate determinations in a representative of three experiments; and

30 Fig. 8 illustrates cellular proliferation in NRK-49F cells transfected with control plasmid or pcKSHV GPCR. After 5 days in culture in medium supplemented with calf serum or fetal calf serum, transfected cells were trypsinized and counted. Cell counts; data represent the mean +/- S.D. of triplicate culture dishes of three transfections.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention provides a constitutively active G protein coupled receptor of human herpesvirus 8.

5        The invention further provides a method of identifying negative antagonists of a constitutively active G protein coupled receptor. The method comprises co-expressing in a host cell a constitutively active G protein coupled receptor and a reporter protein, wherein  
10      expression of the reporter protein is controlled by a promoter responsive to a signalling pathway activated by the constitutively active G protein coupled receptor; exposing the host cell to a test substance; and determining a level of reporter protein activity, wherein  
15      the level of reporter protein activity indicates effectiveness of the test substance as a negative antagonist of the constitutively active G protein coupled receptor.

20      The constitutively active G protein coupled receptor can be any such receptor which one desires to turn off. For example, GPCRs which are tumorigenic or which cause cells to proliferate could be of interest for application of the method of the subject invention. By identifying negative antagonists of such GPCRs, the  
25      negative antagonists could be used to turn off the GPCR and thereby eliminate the GPCR's tumorigenic or cell proliferative effects. An example of such a constitutively active GPCR is the GPCR of human herpesvirus 8 (HHV 8) (also known as Kaposi's sarcoma  
30      associated herpesvirus or KSHV) (Cesarman et al. 1996).

35      Having identified a GPCR of interest, the signalling pathway activated by the GPCR is identified and a promoter responsive to the signalling pathway is selected. Such a "responsive" promoter will only promote expression of a downstream gene if the GPCR is

functioning (i.e., signalling through its pathway). GPCRs use various signalling pathways, including cyclic AMP pathways and protein kinase C pathways. Suitable promoters for use with such GPCRs are cyclic AMP-  
5 responsive promoters and protein kinase C-responsive promoters, respectively. For a review of the cyclic AMP signalling pathway see Habener 1995, and for a review of the protein kinase C signalling pathway see Gershengorn and Perlman 1995.

10 A reporter protein is then chosen. The activity levels of the reporter protein will indicate the levels of GPCR signalling, since the reporter protein will only be expressed if the GPCR is signalling through its pathway to which the selected promoter is responsive.

15 A reporter protein should be chosen which is not generally expressed in the host cell to be utilized for co-expression, to eliminate any false indications of GPCR signalling. A suitable reporter protein, as disclosed in the following examples, is the firefly luciferase gene.

20 The activity of this reporter protein can be readily assayed using a luminescent assay. Other reporter proteins could also be readily used in the method of the subject invention, such as  $\beta$ -galactosidase and growth hormone.

25 Various host cells can be utilized in the method of the subject invention. The choice of a host cell is based on several factors, including the absence of endogenous expression of the reporter protein in the host cell, presence of the GPCR signalling pathway in the host cell, and capability of co-expressing the GPCR and the reporter protein in the host cell. For the analysis of human GPCRs, mammalian cells are preferred, such as COS cells.

30 Generally the co-expression of the constitutively active G protein coupled receptor and the

reporter protein in the host cell will involve introduction of two constructs into the host cell. The first construct is a nucleic acid molecule encoding the G protein coupled receptor under the control of suitable regulatory molecules for expression of the GPCR in the host cell. The second construct is a nucleic acid molecule encoding the reporter protein under the control of the promoter responsive to the signalling pathway of the GPCR. Each construct can be provided in the form of a plasmid, or a single plasmid comprising both constructs could be used. Techniques for introducing the construct(s) into the host cells may involve the use of expression vectors (such as plasmids and viruses) which comprise the nucleic acid molecule encoding the construct(s). DNA encoding the construct can be injected into the nucleus of a host cell or transformed into the host cell using a suitable vector, or mRNA encoding the construct can be injected directly into the host cell, in order to obtain expression of the GPCR and the reporter protein in the host cell.

Various methods are known in the art for introducing nucleic acid molecules into host cells. One method is microinjection, in which DNA is injected directly into the nucleus of cells through fine glass needles (or RNA is injected directly into the cytoplasm of cells). Alternatively, DNA can be incubated with an inert carbohydrate polymer (e.g. dextran) to which a positively charged chemical group (e.g. diethylaminoethyl ("DEAE")) has been coupled. The DNA sticks to the DEAE-dextran via its negatively charged phosphate groups. These large DNA-containing particles, in turn, stick to the surfaces of cells, which are thought to take them in by a process known as endocytosis. Some of the DNA evades destruction in the cytoplasm of the cell and escapes to the nucleus, where it can be transcribed into

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RNA like any other gene in the cell. In another method, cells efficiently take in DNA in the form of a precipitate with calcium phosphate. In electroporation, cells are placed in a solution containing DNA and 5 subjected to a brief electrical pulse that causes holes to open transiently in their membranes. DNA enters through the holes directly into the cytoplasm, bypassing the endocytotic vesicles through which they pass in the DEAE-dextran and calcium phosphate procedures (passage 10 through these vesicles may sometimes destroy or damage DNA). DNA can also be incorporated into artificial lipid vesicles, liposomes, which fuse with the cell membrane, delivering their contents directly into the cytoplasm. In an even more direct approach, DNA is absorbed to the 15 surface of tungsten microprojectiles and fired into cells with a device resembling a shotgun.

Further methods for introducing nucleic acid molecules into cells involve the use of viral vectors. Since viral growth depends on the ability to get the 20 viral genome into cells, viruses have devised clever and efficient methods for doing it. Various viral vectors have been used to transform mammalian cells, such as vaccinia virus, adenovirus, and retrovirus.

As indicated, some of these methods of 25 transforming a cell require the use of an intermediate plasmid vector. U.S. Patent No. 4,237,224 to Cohen and Boyer describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant 30 plasmids are then introduced by means of transformation and replicated in unicellular cultures including prokaryotic organisms and eucaryotic cells grown in tissue culture. The DNA sequences are cloned into the plasmid vector using standard cloning procedures known in 35 the art, as described by Sambrook et al. (1989).

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Various test substances can be screened for their effectiveness as negative antagonists of a constitutively active GPCR. Test substances can be, for example, traditional chemical compounds (such as 5 benzodiazepines) or peptides. Any suitable test substance can be screened in the subject method, as long as the host cell can be exposed to the test substance.

Having thus identified negative antagonists of a constitutively active G protein coupled receptor, and 10 knowing that constitutively active G protein coupled receptors cause tumor formation or cell proliferation, the invention further provides a method of preventing tumor formation or cell proliferation in a subject caused by a constitutively active G protein coupled receptor. 15 The method comprises identifying a negative antagonist of a constitutively active G protein coupled receptor (as discussed above); and administering to the subject an amount of the negative antagonist effective to prevent tumor formation or cell proliferation caused by the constitutively active G protein coupled receptor. In one embodiment, the tumor formation or cell proliferation is caused by a constitutively active G protein coupled receptor of a virus such as human herpesvirus 8.

The following examples relate to the 25 identification of a constitutively active GPCR of human herpesvirus 8, and the assaying of the GPCR activity by co-expressing a reporter protein under the control of a promoter responsive to the GPCR signalling pathway. By then exposing the host cell to a test substance, the 30 effectiveness of the test substance as a negative antagonist of the constitutively active GPCR can be determined (by determining the effect of the test substance on reporter protein activity). The identified negative antagonists could serve as probes to determine 35 the role of agonist-independent HHV8 GPCR activity in

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diseases associated with HHV 8 infections or as lead compounds for drugs (Navia and Murcko 1992) if a role for HHV8 GPCR in a disease process(es) is demonstrated. In particular, because constitutively active GPCRs have been 5 found to be tumorigenic in mice (Young et al. 1986) or to mediate proliferation or transformation of cells infected with HHV 8 (Julius et al. 1989; Allen et al. 1991; Gutkind et al. 1991), these negative antagonists could lead to the development of anti-tumor drugs.

10

MATERIALS AND METHODS

**Competition binding.** The coding sequence of KSHV GPCR was cleaved from its original subcloning 15 plasmid (Cesarman et al. 1996) by digestion with NcoI and inserted into the EcoRV site of the eukaryotic expression vector pcDNA3.1(+) (Invitrogen), producing the pcKSHV GPCR used for expression in mammalian cells. COS-1 cells (American Type Culture Collection) were grown in 100 mm 20 petri dishes in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) with 5% Nu-Serum (Collaborative Research) in a humidified incubator in an atmosphere of 5% CO<sub>2</sub> at 37°C. DEAE-dextran transfections were carried out as previously described (Nussenzveig et al. 1994). COS-1 cells were 25 transfected with 10 mg/ml of pcKSHV GPCR and tested for IL-8 binding 48 hrs post-transfection. Approximately 100,000 cells per well (in 12-well plates) were washed with Hank's balanced salt solution (HBSS) containing 25 mM HEPES, pH 7.4 (GIBCO-BRL). Between 0.3 and 0.5 nM 30 <sup>125</sup>I-IL-8 (DuPont-NEN) in the absence or presence of various concentrations of unlabelled chemokines was added and binding was allowed to proceed at 4°C for 2 hrs with gentle rocking. The binding buffer contained bovine serum albumin (BSA; 1 mg/ml), bacitracin (1 mg/ml), and 35 phenyl methyl sulphonyl fluoride (PMSF; 1mM). Assays

were terminated by aspirating the binding buffer, washing three times with chilled HBSS and solubilizing the cells with 0.4 N NaOH. An aliquot of this solution was counted in a gamma counter and the data expressed as percent of 5 maximum bound. Binding and dose-response curves were analyzed using Prism software (GraphPad, Inc.).

**Inositol phosphate accumulation.** COS-1 cells were transfected with pcKSHV GPCR or plasmids encoding human IL-8 receptor types A or B and either Ga<sub>16</sub> or Ga<sub>15</sub> 10 (Wu et al. 1993). All transfection cocktails were supplemented to contain equal total plasmid DNA by the addition of appropriate amounts of vector (pcDNA3.1). Twenty four hrs following transfection, cells were harvested and reseeded in 12-well plates in DMEM with 10 15 % Nu serum and 1 mCi/ml myo-[<sup>3</sup>H]inositol (DuPont-NEN), and maintained in culture as before for an additional 24 hrs. After removing the culture media and rinsing the cells, cells were incubated in HBSS containing 25 mM HEPES, pH 7.4 with 10 mM LiCl in the absence or presence of human 20 recombinant IL-8 (R&D Systems) at 0.25 mg/ml. Cells were harvested after 90 min or other time points, and IP accumulation measured using ion exchange chromatography as described previously (Straub et al. 1990).

**Luciferase assay.** Cells were transfected with 25 increasing amounts of pcKSHV-GPCR (0.003 to 0.100 mg/ml) and 5 mg/ml pAP1-fos-LUC (Schadlow et al. 1992), supplemented to contain equal total plasmid DNA by the addition of appropriate amounts of vector (pcDNA3.1). Cells were washed with phosphate-buffered saline (PBS) 30 and lysed with 0.5 ml/10 cm dish of lysis buffer (25 mM GlyGly, 15 mM MgSO<sub>4</sub>, 4 mM EGTA, 1 mM dithiothreitol (DTT), 1% Triton X-100, pH 7.8). Cell extracts were diluted 1:10 or 1:20 in reaction buffer (25 mM GlyGly, 15 mM MgSO<sub>4</sub>, 4 mM EGTA, 1 mM DTT, 15 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM ATP, pH

7.8), combined with equal volumes of 0.4 mM luciferin in reaction buffer and luminescence measured for 10 sec.

Cell proliferation assay. "Normal rat kidney" fibroblasts (NRK-49F; ATCC) were seeded at  $2 \times 10^5$  / 100 mm dish, grown in DMEM with 10% fetal calf serum, and transfected with control vector (20 mg/plate), pcKSHV GPCR (20 mg/plate) or pRas-Zip (10 mg/plate; positive control) (Dotto et al. 1985) using the calcium phosphate method as previously described (Loyter et al. 1982).  
Cells were harvested 24 hrs post-transfection and seeded at a density of 5,000 cells / 100 mm dish in DMEM supplemented with either 10 % calf serum or fetal calf serum. On day 5 post-transfection, cells were photographed for qualitative assessment (Nikon inverted phase microscope) and then trypsinized to determine total cell number using a hemocytometer.

**EXAMPLE I**

20 Kaposi's sarcoma-associated herpesvirus (KSHV)/human herpesvirus 8 (HHV 8) is a recently described virus consistently present in Kaposi's sarcoma (KS) (Chang et al. 1994; Moore and Chang 1995) and primary effusion (body cavity-based) lymphomas (PEL) (Cesarman et 25 al. 1995) which are malignancies occurring frequently, but not exclusively, in AIDS patients. KSHV is a gammaherpesvirus with homology to Herpesvirus Saimiri (HVS) and Epstein-Barr virus (EBV) (Chang et al. 1995; Moore et al. 1996), both of which are viruses capable of 30 transforming lymphocytes (Roizman 1996). Cloning of a viral genome fragment revealed the presence of an open reading frame encoding a putative G protein-coupled receptor (KSHV GPCR) (Cesarman et al. 1996) that is homologous to a GPCR encoded by HVS (Nicholas et al. 35 1992; Ahuja and Murphy 1993) and to human interleukin 8

(IL-8) receptors (Holmes et al. 1991; Murphy and Tiffany 1991). In the studies that follow, it is shown that KSHV GPCR is a *bona fide* signaling receptor with constitutive (agonist-independent) activity via the

5 phosphoinositide-1,4,5 inositoltrisphosphate-protein kinase C (PKC) cascade. Furthermore, KSHV GPCR is shown to stimulate cellular proliferation, making it a candidate viral oncogene. The nucleic acid and amino acid sequences of the HHV 8 GPCR are disclosed in co-pending, co-assigned U.S. Serial No. 08/728,603, filed 10 October 10, 1996, the contents of which are hereby incorporated by reference.

Fig. 1 illustrates the putative two-dimensional topology of HHV 8 GPCR. The top of the diagram represents the extracellular space (E), the middle portion represents the transmembrane (TM) domain, and the bottom portion represents the intracellular space (C). Each circle represents an amino acid residue designated by the single letter code.

As is evident, HHV 8 GPCR can be represented with the signature seven TM-spanning domains of all GPCRs. This, however, is not sufficient to classify this protein as a receptor. Moreover, it is lacking some of the residues that are highly conserved in GPCRs. The most relevant to this application relate to amino acid residues that have been found to be involved in activation of other GPCRs. For example, an Asp residue is usually present in putative TM-2 that has been shown to be critical for activation of a number of GPCRs (Baldwin 1994). There is an Asp at this position in both IL8-Rs but not in the GPCR in the Herpesvirus saimiri genome and not in HHV 8 GPCR. In HHV 8 GPCR this Asp has been replaced by Asn. A similar replacement of Asp by Asn in TM-2 is present in the receptor for 20 gonadotropin-releasing hormone (GnRH) but in the GnRH 25

receptor there is a complementary change of a highly conserved Asn in TM-7, which is in a Asn-Pro-Xaa-Xaa-Tyr motif (Baldwin 1993), to Asp (Zhou et al. 1994) that is not present in HHV 8 GPCR. It has been postulated that 5 interactions between these two residues, Asp and Asn, may be necessary for receptor activation (Van Rhee and Jacobson 1996). There is a Val at this position in TM-7 in HHV 8 GPCR that will not be able to form a hydrogen bond with the Asn in TM-2. The absence of this potential 10 interaction between TM-2 and TM-7 may render HHV 8 GPCR constitutively active, but this cannot be predicted from the sequence.

A number of experiments were performed to show that HHV 8 GPCR is a functioning receptor. To 15 investigate whether the KSHV GPCR gene encodes a functional receptor, the KSHV GPCR was expressed in COS cells and a determination whether the receptor binds IL-8 and other chemokines was made. IL-8, which is a member of the family of C-X-C chemokines (Baggiolini et al. 1994), was chosen as radioligand because of the homology 20 of KSHV GPCR to cellular IL-8 receptors. Furthermore, since KS lesions are sites of active inflammation and have been shown to produce IL-8 and other cytokines (Sciacca et al. 1994), it is possible that such areas 25 could provide stimulatory signals for the KSHV GPCR. The binding characteristics of KSHV GPCR were investigated using  $^{125}\text{I}$ -IL-8 and various concentrations of other chemokines in a competition analysis. As shown in Fig. 2, KSHV GPCR binds IL-8 with an apparent equilibrium 30 dissociation constant ( $K_d$ ) of 30 nM, which is lower affinity than the values previously reported for binding of IL-8 to the human IL-8 receptor types A or B ( $K_d$  of 0.8-1.0 nM) (Holmes et al. 1991; Murphy and Tiffany 1991). Since chemokine receptors may bind more than one ligand, 35 several other C-X-C chemokines were tested, namely MGSA,

NAP-2 and PF-4 (Baggiolini et al. 1994), and it was found that they also bind to KSHV GPCR (Fig. 2). Although chemokines of the C-C family do not bind to IL-8 receptors (Baggiolini et al. 1994), it was found that 5 I-309 and RANTES bind to KSHV GPCR whereas MIP-1b and MCP-1 do not (Fig. 3). The rank order of binding was: IL-8 = NAP-2 > PF-4 = I-309 = MGSA > RANTES >> MIP-1b and MCP-1. Thus, KSHV GPCR exhibits binding characteristics of a chemokine receptor with affinity for a range of 10 chemokines spanning the C-X-C and C-C families.

To assess whether KSHV GPCR is a signal transducing receptor, the formation of intracellular second messengers in COS cells transiently expressing KSHV GPCR was measured. Inositol phosphate (IP) 15 accumulation was measured because IL-8 receptors signal via the phosphoinositide-IP-calcium/diacylglycerol-PKC cascade (Holmes et al. 1991; Murphy and Tiffany 1991). Stimulated IP formation can be measured in COS cells co-expressing IL-8 receptors and the  $\alpha$  subunits of the 20 heterotrimeric proteins  $G_{\alpha 16}$  ( $G_{\alpha 16}$ ) or  $G_{\alpha 15}$  ( $G_{\alpha 15}$ ) in the presence but not in the absence of IL-8 (Wu et al. 1993). In agreement with that report, little measurable 25 accumulation of IPs in COS cells expressing IL-8 receptors in the absence or presence of IL-8 was found, unless  $G_{\alpha 16}$  or  $G_{\alpha 15}$  was cotransfected (Fig. 4). In cells expressing IL-8 receptors and  $G_{\alpha 16}$  or  $G_{\alpha 15}$ , IL-8 stimulated IP formation. In contrast, and under these experimental 30 conditions, cells transiently expressing KSHV GPCR showed IP accumulation which was higher than that obtained with human IL-8 receptors activated by IL-8. In cells transiently expressing KSHV GPCR, IP formation was elevated in the absence of added ligand and without co-expression of  $G_{\alpha 16}$  or  $G_{\alpha 15}$ , and was not increased by 35 IL-8 or any of the chemokines that showed binding to KSHV GPCR. There was a constant rate of IP accumulation for

at least 4 hours (Fig. 5); IL-8 had no effect at any time point. Transfection of COS-1 cells with increasing amounts of plasmid encoding KSHV GPCR showed that IP accumulation was directly proportional to the amount of 5 plasmid used for transfection (between 0.1 and 3 mg/ml plasmid) (Fig. 6). Again, there was no effect of IL-8 on IP accumulation at any level of KSHV GPCR expression. Thus, KSHV GPCR exhibits agonist-independent (or 10 constitutive) activity in COS-1 cells. In contrast to 15 IL-8 receptors, KSHV GPCR couples effectively to the G protein(s) endogenous in COS-1 cells. Since IP accumulation from the KSHV GPCR is resistant to pertussis toxin, this suggests that the receptor may signal through the Gq family of G-proteins.

20 To determine whether the constitutive signaling activity of KSHV GPCR leads to stimulation of downstream processes, the effect of basal second messenger formation on gene transcription was measured. A reporter gene, firefly luciferase, under the control of a PKC-responsive promoter containing an AP-1 binding motif (Schadlow et al. 1992) was used to measure activity of the 25 diacylglycerol/PKC limb of the phosphoinositide signaling pathway. In these experiments, COS-1 cells were co-transfected with plasmid encoding KSHV GPCR and a 30 plasmid containing a minimal promoter derived from the human c-fos gene to which a PKC-responsive element was inserted to drive luciferase transcription (Schadlow et al. 1992). As shown in Fig. 7, there was a direct 35 relationship between the amount of plasmid encoding KSHV GPCR used for transfection and the level of luciferase activity. Thus, second messenger formation caused by the basal activity of KSHV GPCR is sufficient to induce gene transcription for a PKC-responsive promoter. This finding is important given the wide range of genes, including those for inflammatory and angiogenic agents,

whose transcription is regulated by PKC-responsive promoters (Karin 1995). Of note is the observation that luciferase activity (Fig. 7) is stimulated at lower plasmid levels than IP accumulation (Fig. 6). This 5 likely occurred because induction of gene transcription is a more amplified response occurring downstream of second messenger formation.

EXAMPLE II

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To test whether the constitutive KSHV GPCR signaling activity affects cellular proliferation, NRK-49F cells were transiently transfected with plasmid encoding KSHV GPCR and monitored for proliferation as 15 compared to cells transfected with control vector (negative control) or with a plasmid encoding the Ras oncogene (positive control) (Dotto et al. 1985). Culture dishes transfected with Ras-encoding plasmids contained more cells than control dishes within 24 to 48 hrs. As 20 early as 72 hrs post-transfection, the KSHV GPCR transfectants exhibited increased cell number as compared to the control transfectants. This response was confirmed by phase contrast microscopy and quantified after 5 days in culture (Fig. 8). All clusters 25 transfected with pcKSHV GPCR contained many more, closely apposed cells than clusters of cells transfected with control plasmid.

Since gammaherpesviruses cause transformation (Roisman 1996) and GPCRs have been found to be oncogenic 30 under some circumstances (Julius et al. 1989), it is possible that KSHV may be a transforming agent that acts, at least in part, through KSHV GPCR. This possibility is further strengthened by the specific association of KSHV with KS and PEL, and the fact that KSHV GPCR is expressed 35 at the RNA level in both of these types of malignancies

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(Cesarman et al. 1996; Arvanitakis et al. 1996). These results have now demonstrated that KSHV GPCR is a bona fide receptor that signals constitutively via the phosphoinositide transduction pathway. Although it has 5 an amino acid sequence that is homologous to IL-8 receptors, binds IL-8 with relatively high affinity and activates the same signal transduction cascade, KSHV GPCR has two characteristics that make it a more promiscuous receptor and one more likely to be involved in 10 pathogenesis than IL-8 receptors. KSHV GPCR signals constitutively, that is, it does not require activation by an agonist(s) and is capable of coupling to a wider variety of G proteins than IL-8 receptors. Coupling of KSHV GPCR to G proteins other than  $G_{\alpha 16}$ , as shown by 15 signaling in COS cells that do not express this G protein, would allow KSHV GPCR to signal in a broader range of cell types. Constitutive signaling activity, which is exhibited by some native GPCRs (Milano et al. 1994), has been observed more commonly with mutated GPCRs 20 (Lefkowitz et al. 1993; Alblas et al. 1996) in an increasing number of human diseases, including tumors (Coughlin 1994; Sande et al. 1995). Finally, the enhanced proliferation exhibited by KSHV GPCR transfectants supports a role for this receptor in 25 tumorigenesis.

The presence of this functional, constitutively active GPCR in the genome of a virus that is so tightly associated with specific malignancies provides circumstantial evidence toward its pathogenetic role. 30 Two alternative, or perhaps complementary, hypotheses can be proposed to explain the role of the viral GPCR in the development of KSHV-associated diseases: a direct mechanism, in which the constitutive signaling activity of KSHV GPCR leads to altered growth and neoplastic 35 transformation, and/or an indirect mechanism, where

signaling by KSHV GPCR induces the expression of other genes which are in turn involved in pathogenetic events. For example, the latter model could apply to the case of basic fibroblast growth factor (bFGF) which has 5 previously been shown to play a pivotal role in the development of KS lesions (Ensoli et al. 1989), and whose expression is driven by an AP-1 binding, and probably PKC-responsive promoter (Erdos et al. 1995).

The determination of the effect on cells 10 expressing HHV 8 GPCRs of negative antagonists of HHV 8 GPCRs will delineate the role(s) of HHV 8 GPCRs in infected cells. In this regard, the two most important downstream effects are the stimulation of cell proliferation or if HHV 8 GPCR activity caused cell 15 transformation. One can determine whether HHV 8 GPCR can induce cell transformation and whether antagonists can inhibit this effect. Transformation in NIH 3T3 fibroblasts can be used in a protocol used to show that the agonist-activated serotonin 1c receptor, another 20 GPCR, is capable of triggering transformation (Julius et al. 1989). In this assay, HHV 8 GPCR expression will be produced by transfection with plasmids encoding HHV 8 GPCR, not by infection with HHV-8. This assay will show whether HHV 8 GPCR expression leads to a transformed 25 phenotype and provide a quantitative assessment of the effect of antagonist on transforming activity of HHV 8 GPCR because the number of foci of transformed cells in the absence versus the presence of antagonist can be counted. One can also determine whether HHV-8 infection 30 will transform CD19<sup>+</sup> B-cells in experiments similar to those performed to show lymphoblastoid transformation by Herpesvirus saimiri and Epstein-Barr viruses (Rangan et al. 1977; Miller 1974). If this series of experiments shows that HHV 8 GPCR expression causes transformation in 35 these *in vitro* assays, one can determine whether cells

from these foci will generate tumors in nude mice and whether this can be inhibited by antagonists.

B-cell lines will be used for studies of cell proliferation because of ease of their experimental manipulation. These B-cell lines may not be good models for study of proliferation because they may have acquired other changes (mutations) that allow them to proliferate without the signalling activity of HHV 8 GPCRs. Acutely infected cells, such as B-cells, will be used and one can study other cell lines including human lymphoblastoid cell lines UH-1 and CB-33, and endothelial and fibroblast cell lines derived from KS lesions. An effect of antagonists of HHV 8 GPCR to inhibit the rates of cell proliferation will be monitored. This will be tested in a standard assay by measuring the rate of proliferation of cells incubated without (control) and with antagonists. By counting cells, which are seeded at low density, over time, one can determine the effect of these antagonists on the generation times of these cells.

Different incubation mediums can be tested and, in particular, attempt to adjust the type and concentration of the serum supplement so that there is a moderate generation time for cells in the absence of antagonists. This will allow for the highest likelihood of observing an inhibitory effect on proliferation. These experiments will be complemented with those that measure DNA synthesis more directly, for example, by measuring [<sup>3</sup>H]thymidine incorporation into DNA and by measuring DNA content, for example with 5-bromodeoxyuridine. The most definitive measurement of cell proliferation is, however, the rate of increase in cell number over time.

These experiments will establish whether HHV 8 GPCR is expressed at the protein level in infected cells, whether HHV 8 GPCR stimulates cellular transformation and/or proliferation and whether the negative antagonists

discovered according to the method of the subject invention will inhibit these effects.

Although preferred embodiments have been depicted and described in detail herein, it will be 5 apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which 10 follow.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Cornell Research Foundation, Inc.

(ii) TITLE OF INVENTION: CONSTITUTIVELY ACTIVE G PROTEIN COUPLED RECEPTOR OF HHV 8 AND METHOD OF IDENTIFYING NEGATIVE ANTAGONISTS OF G PROTEIN COUPLED RECEPTORS

(iii) NUMBER OF SEQUENCES: 1

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- (D) STATE: New York
- (E) COUNTRY: U.S.A.
- (F) ZIP: 14603

## (v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

## (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

## (vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/785,928
- (B) FILING DATE: 22-JAN-1997

## (viii) ATTORNEY/AGENT INFORMATION:

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- (B) REGISTRATION NUMBER: 30,727
- (C) REFERENCE/DOCKET NUMBER: 19603/1321

## (ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (716) 263-1304
- (B) TELEFAX: (716) 263-1600

## (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 342 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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20 25 30

Ser Leu Glu Val Ser Val Cys Glu Met Thr Thr Val Val Pro Tyr Thr  
35 40 45

Trp Asn Val Gly Ile Leu Ser Leu Ile Phe Leu Ile Asn Val Leu Gly  
50 55 60

Asn Gly Leu Val Thr Tyr Ile Phe Cys Lys His Arg Ser Arg Ala Gly  
65 70 75 80

Ala Ile Asp Ile Leu Leu Gly Ile Cys Leu Asn Ser Leu Cys Leu  
85 90 95

Ser Ile Ser Leu Leu Ala Glu Val Leu Met Phe Leu Phe Pro Asn Ile  
100 105 110

Ile Ser Thr Gly Leu Cys Arg Leu Glu Ile Phe Phe Tyr Tyr Leu Tyr  
115 120 125

Val Tyr Leu Asp Ile Phe Ser Val Val Cys Val Ser Leu Val Arg Tyr  
130 135 140

Leu Leu Val Ala Tyr Ser Thr Arg Ser Trp Pro Lys Lys Gln Ser Leu  
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Gly Trp Val Leu Thr Ser Ala Ala Leu Leu Ile Ala Leu Val Leu Ser  
165 170 175

Gly Asp Ala Cys Arg His Arg Ser Arg Val Val Asp Pro Val Ser Lys  
180 185 190

Gln Ala Met Cys Tyr Glu Asn Ala Gly Asn Met Thr Ala Asp Trp Arg  
195 200 205

Leu His Val Arg Thr Val Ser Val Thr Ala Gly Phe Leu Leu Pro Leu  
210 215 220

Ala Leu Leu Ile Leu Phe Tyr Ala Leu Thr Trp Cys Val Val Arg Arg  
225 230 235 240

Thr Lys Leu Gln Ala Arg Arg Lys Val Arg Gly Val Ile Val Ala Val  
245 250 255

- 28 -

Val Leu Leu Phe Phe Val Phe Cys Phe Pro Tyr His Val Leu Asn Leu  
260 265 270

Leu Asp Thr Leu Leu Arg Arg Arg Trp Ile Arg Asp Ser Cys Tyr Thr  
275 280 285

Arg Gly Leu Ile Asn Val Gly Leu Ala Val Thr Ser Leu Leu Gln Ala  
290 295 300

Leu Tyr Ser Ala Val Val Pro Leu Ile Tyr Ser Cys Leu Gly Ser Leu  
305 310 315 320

Phe Arg Gln Arg Met Tyr Gly Leu Phe Gln Ser Leu Arg Gln Ser Phe  
325 330 335

Met Ser Gly Ala Thr Thr  
340

WHAT IS CLAIMED IS:

1. A method of identifying negative antagonists of a constitutively active G protein coupled receptor, said method comprising:

co-expressing in a host cell a constitutively active G protein coupled receptor and a reporter protein, wherein expression of said reporter protein is controlled by a promoter responsive to a signalling pathway activated by the constitutively active G protein coupled receptor;

exposing said host cell to a test substance; and

determining a level of reporter protein activity, wherein the level of reporter protein activity indicates effectiveness of the test substance as a negative antagonist of said constitutively active G protein coupled receptor.

20 2. The method of claim 1 wherein said promoter is a cyclic AMP-responsive promoter.

25 3. The method of claim 1 wherein said promoter is a protein kinase C-responsive promoter.

4. The method of claim 1 wherein said reporter protein is luciferase.

30 5. The method of claim 4 wherein determining a level of luciferase activity uses a luminescent assay.

6. The method of claim 1 wherein said host cell is a mammalian cell.

7. The method of claim 6 wherein said mammalian cell is a COS cell.

5 8. The method of claim 1 wherein said constitutively active G protein coupled receptor is a G protein coupled receptor of human herpesvirus 8.

10 9. A method of preventing tumor formation or cell proliferation in a subject caused by a constitutively active G protein coupled receptor, said method comprising:

15 identifying a negative antagonist of a constitutively active G protein coupled receptor; and administering to the subject an amount of the negative antagonist effective to prevent tumor formation or cell proliferation caused by the constitutively active G protein coupled receptor.

20 10. The method of claim 9 wherein said tumor formation or cell proliferation is caused by a constitutively active G protein coupled receptor of a virus.

25 11. The method of claim 10 wherein said virus is a human herpesvirus 8.

30 12. The method of claim 9 wherein said identification of a negative antagonist of a constitutively active G protein coupled receptor comprises:

co-expressing in a host cell a constitutively active G protein coupled receptor and a reporter protein, wherein expression of said reporter protein is controlled by a promoter responsive to a

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signalling pathway activated by the constitutively active G protein coupled receptor;

exposing said host cell to a test substance; and

5 determining a level of reporter protein activity, wherein the level of reporter protein activity indicates effectiveness of the test substance as a negative antagonist of said constitutively active G protein coupled receptor.

10

13. The method of claim 12 wherein said promoter is a cyclic AMP-responsive promoter.

15

14. The method of claim 12 wherein said promoter is a protein kinase C-responsive promoter.

15. The method of claim 12 wherein said reporter protein is luciferase.

20

16. The method of claim 15 wherein determining a level of luciferase activity uses a luminescent assay.

17. The method of claim 12 wherein said host cell is a mammalian cell.

25

18. The method of claim 17 wherein said mammalian cell is a COS cell.

30

19. The method of claim 12 wherein said constitutively active G protein coupled receptor is a G protein coupled receptor of human herpesvirus 8.

20. A constitutively active G protein coupled receptor of human herpesvirus 8.

35

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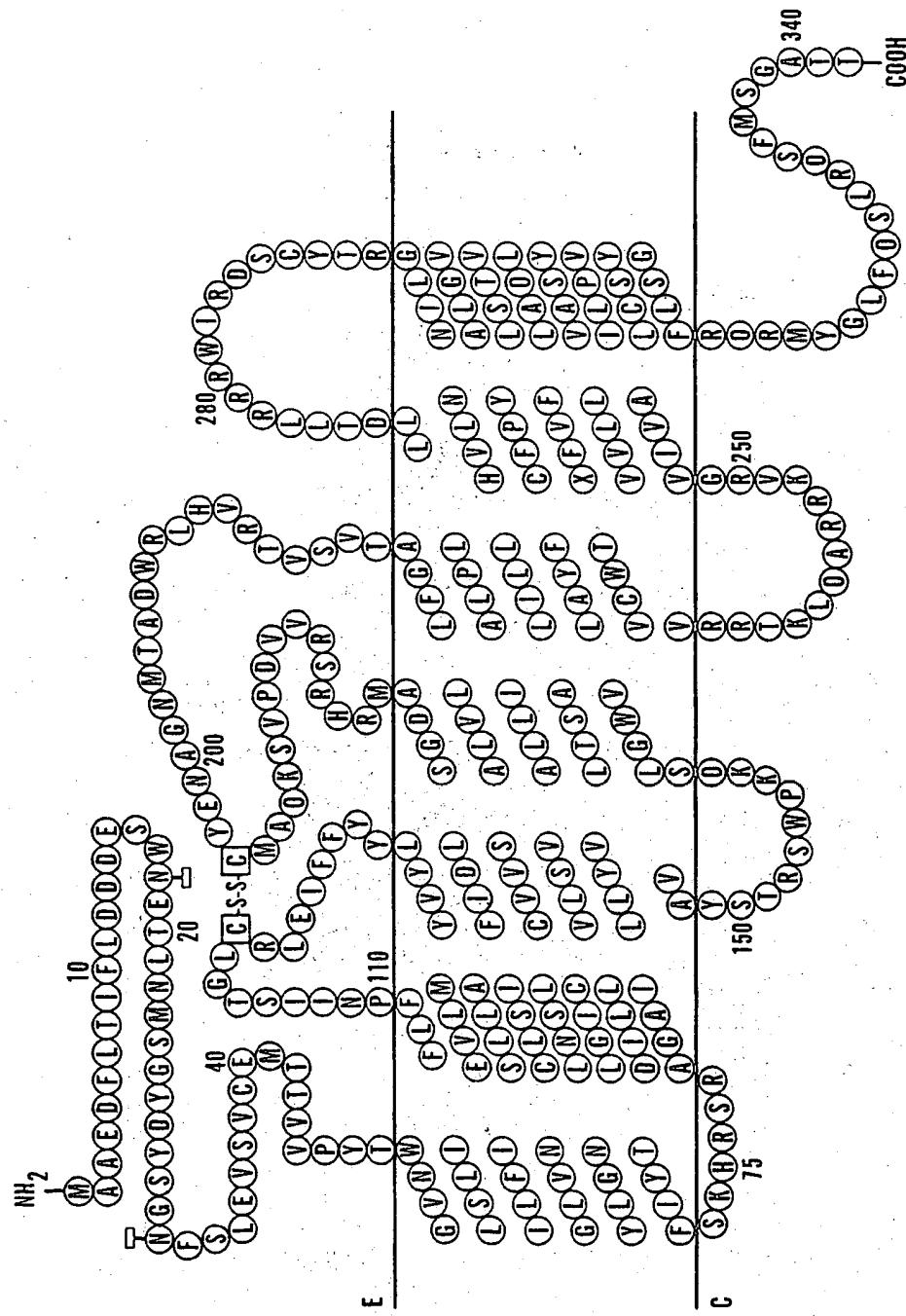
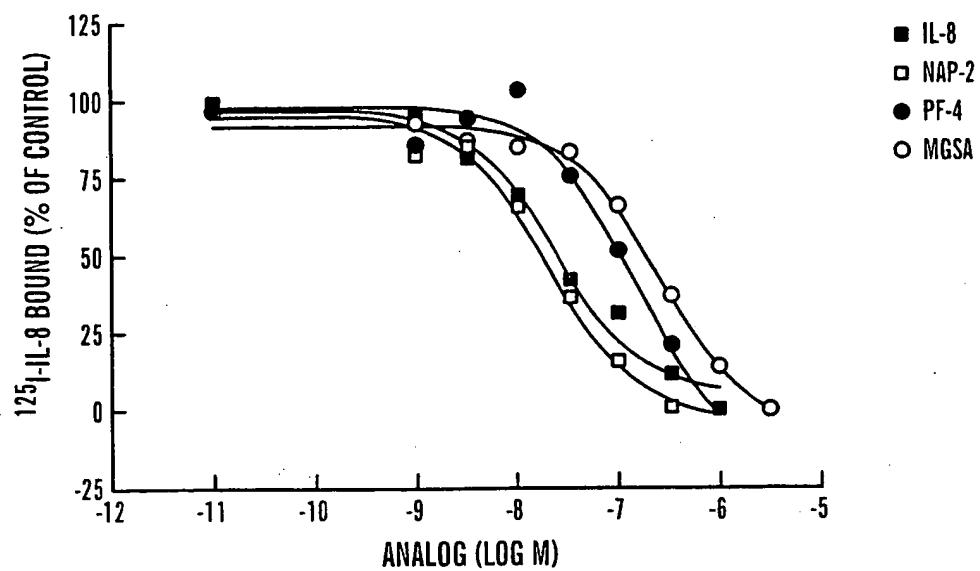
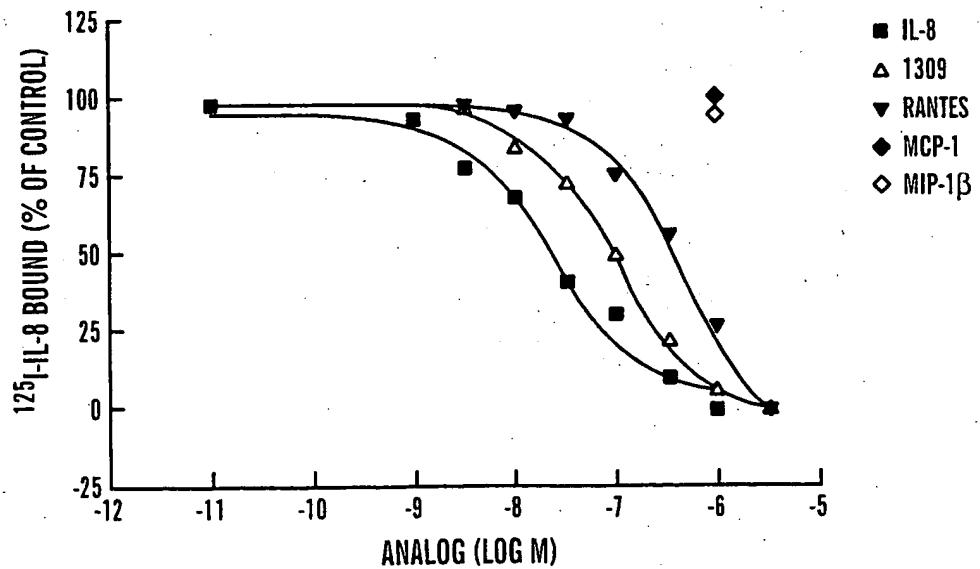


FIG. 1

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**FIG. 2****FIG. 3**

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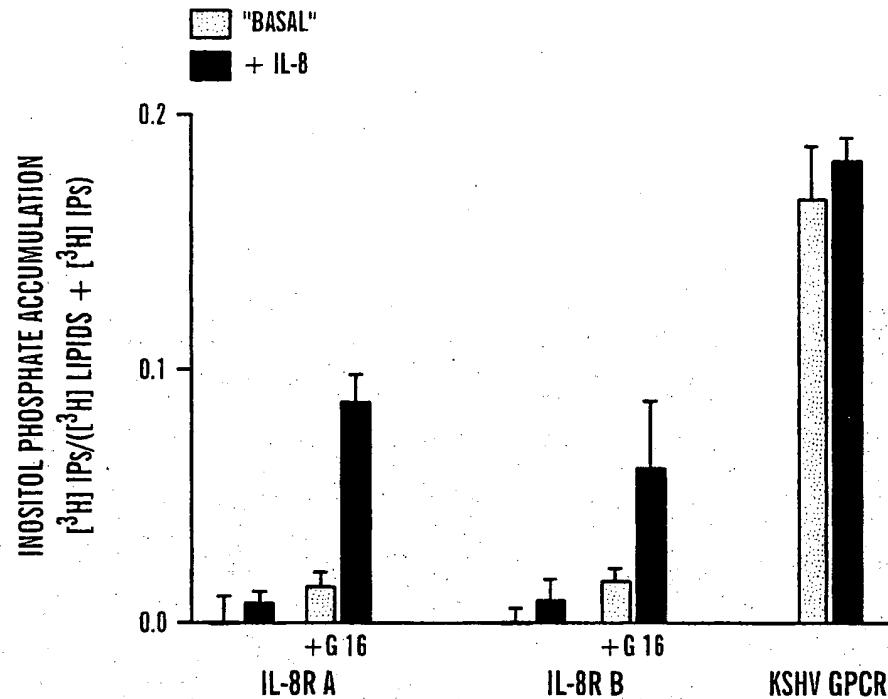


FIG. 4

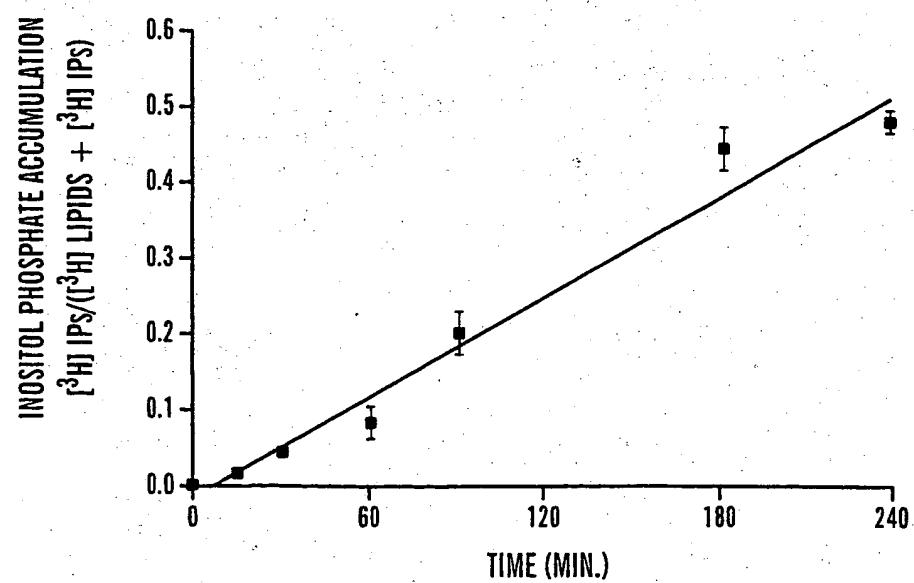


FIG. 5

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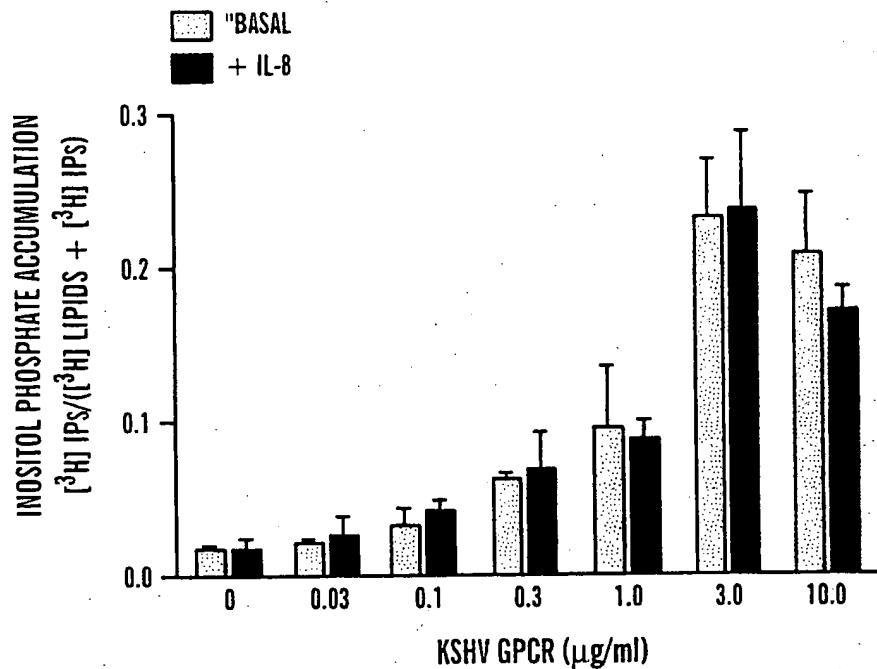


FIG. 6

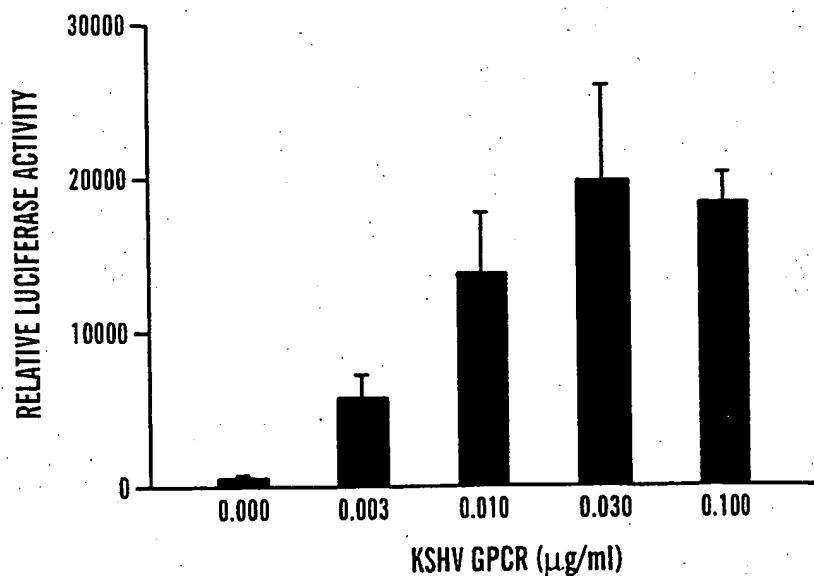
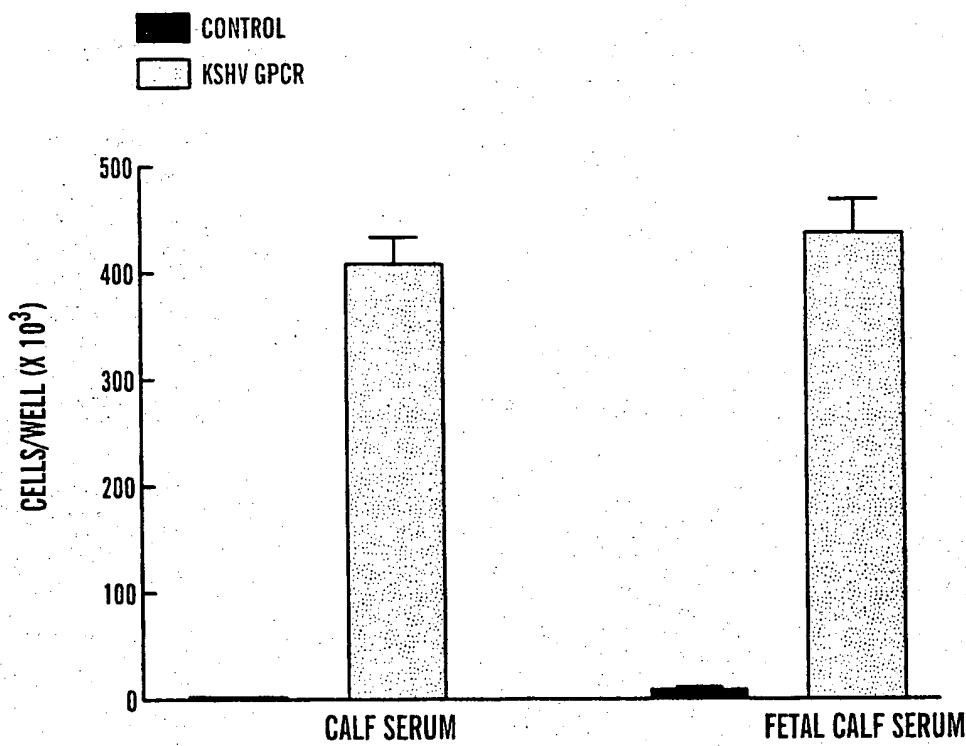


FIG. 7

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**FIG. 8**

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/01089

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/02; C07K 14/705; C12N 15/12, 15/70, 15/79, 15/81

US CL :435/7.2, 69.1, 325, 252.3, 254.11; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.2, 69.1, 325, 252.3, 254.11; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Biosis, Medline, WPI

search terms: constitutively active G-protein coupled receptor, negative antagonist, human herpesvirus 8

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CESAR MAN et al. Kaposi's Sarcoma-Associated Herpesvirus Contains G Protein-Coupled Receptor and Cyclin D Homologs which are Expressed in Kaposi's Sarcoma and Malignant Lymphoma. Journal of Virology. November 1996, Vol. 70, No. 11, pages 8218-8223, especially pages 8219 and 8220.	1-8, 20
Y	EGGERICKX et al. Molecular Cloning of an Orphan G-Protein-Coupled Receptor that Constitutively Activates Adenylate Cyclase. Biochem. J. 1995, Vol. 309, pages 837-843, especially pages 838, 840, and 841.	1-8, 20

 Further documents are listed in the continuation of Box C.

See patent family annex.

•	Special categories of cited documents:	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A*	document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E*	earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L*	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
*O*	document referring to an oral disclosure, use, exhibition or other means		
*P*	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
02 APRIL 1998	18 MAY 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer <i>Sally P. Teng</i>
Faximile No. (703) 305-3230	Telephone No. (703) 308-0196

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US98/01089

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HEINFLINK et al. A Constitutively Active Mutant Thyrotropin-Releasing Hormone Receptor is Chronically Down-Regulated in Pituitary Cells: Evidence Using Clordiazepoxide as a Negative Antagonist. Molecular Endocrinology. 1995, Vol. 9, No. 11, pages 1455-1460, especially pages 1456-1458.	1-8, 20
Y	US 5,436,128 A (HARPOLD et al) 25 July 1995, columns 24, 25, and 30-34.	1-8, 20
Y	US 5,462,856 A (LERNER et al) 31 October 1995, columns 15-17 and 19-22.	1-8, 20

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/01089

**Box 1 Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US98/01089

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-8 and 20, drawn to a method of identifying negative antagonists of a constitutively active G-protein coupled receptor and to a constitutively active G-protein coupled receptor.

Group II, claims 9-19, drawn to a method of preventing tumor formation or cell proliferation.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical features of Group I are a method of identifying negative antagonists of a constitutively active G-protein coupled receptor and the structure of the constitutively active G-protein coupled receptor. The special technical feature of Group II is a method of preventing tumor formation or cell proliferation by administering a negative antagonist. The special technical feature of each group is not the same or does not correspond to the special technical feature of any other group because the methods of groups I and II require different starting material and method steps for achieving different goals. The groups are not linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.